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extensive mortality of invertebrates. Recolonization of contaminated sediment occurred rapidly but recolonization was by different benthic invertebrate species than recolonized uncontaminated sediment. Amphipods showed changes in feeding and movement activities when exposed to sediment contaminated with fresh Prudhoe Bay crude oil. Amphipods selectively burrowed into non-oil contaminated sediment even after the oil had weathered. Oil utilizing microorganisms comprised a high proportion of the heterotrophic microorganisms in the flow through system exposed to crude oil and in sediment in the freshwater lake impacted by the 1976 NARL gasoline spillage. The composition of the microbial community indicated that the effects of the NARL gasoline spillage were widespread in the lake and were persistent. Numerical taxonomic studies showed that similar organisms dominated the hydrocarbon utilizing populations in the water and sediment samples from the gasoline impacted area of the freshwater lake and in water samples from the flow-through system exposed to Prudhoe crude oil. Results indicate that both microbial seeding and application of oleophilic fertilizers may be valuable in accelerating biodegradation of petroleum hydrocarbons in Arctic ecosystems.

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Fate and Effects of Oil Pollutants in Extremely Cold Marine Environments

by

R.M. Atlas

Department of Biology
University of Louisville
Louisville, Kentucky 40208

December 31, 1977

ABSTRACT

Petroleum degradation was studied using Prudhoe Bay crude oil incubated In situ in several Arctic ecosystems. Studies were conducted in water, ice and sediment ecosystems. No biodegradation of oil was found when oil was allowed to freeze into the ice matrix. When oil was spilled under ice evaporative losses were greatly restricted and biodegradation rates were very low. In sediment, biodegradative changes in the oil were found within a few days of spillage. When sediment was contaminated with oil there was extensive mortality of invertebrates. Recolonization of contaminated sediment occurred rapidly but recolonization was by different benthic invertebrate species than recolonized uncontaminated sediment. Amphipods showed changes in feeding and movement activities when exposed to sediment contaminated with fresh Prudhoe Bay crude oil. Amphipods selectively burrowed into non-oil contaminated sediment even after the oil had weathered. Oil utilizing microorganisms comprised a high proportion of the heterotrophic microorganisms in the flow through system exposed to crude oil and in sediment in the freshwater lake impacted by the 1976 NARL gasoline spillage. The composition of the microbial community indicated that the effects of the NARL gasoline spillage were widespread in the lake and were persistent. Numerical taxonomic studies showed that similar organisms dominated the hydrocarbon utilizing populations in water and sediment from the gasoline impacted area of the freshwater lake and water from the flow-through system exposed to Prudhoe crude oil. Results indicate that both microbial seeding and application of oleophilic fertilizers may be valuable in accelerating biodegradation of petroleum hydrocarbons in Arctic ecosystems.

INTRODUCTION

Several *In situ* model systems were used to study the fate of oil in Arctic ecosystems. Oil may contaminate water, ice or sediment. Both marine and terrestrial ecosystems may be contaminated. Long term studies are required to assess the fate and effects of contaminating oil in Arctic ecosystems.

Previous flow through oil degradation studies were extended for almost 1 year. This extends the previous studies to include changes in the oil that occur during winter. The fate of oil under ice during spring was once again examined. A long term *In situ* oil in sediment study was begun. This study is designed to follow the degradative changes in the oil and the effects of the oil on the benthic community. Laboratory studies were also extended to determine the sublethal effects of oil in sediment on Arctic amphipods.

Monitoring studies of the freshwater lake at NARL contaminated by an accidental leaded gasoline spillage were continued. This actual spillage is being used to study the ability of microbial community to degrade the contaminating gasoline and the changes in the microbial community caused by the input of the gasoline. Changes in the microbial community can be used to monitor the extent of gasoline contamination and the persistence of the effects of the contamination.

Numerical taxonomic studies were conducted on the microorganisms from the oil-flow through system and the gasoline contamination areas.

These studies are intended to determine the dominant microflora that flourish following contamination of Arctic ecosystems with petroleum hydrocarbons.

MATERIALS AND METHODS

Continuous Flow System

The continuous open flow-through system previously described (Horowitz and Atlas, 1977a) was modified by eliminating the second chamber and using rubber tubing to regulate the water level in the primary chamber. The study was begun in July 1976. Temperature, salinity, oxygen concentration, pH, ammonia concentrations, nitrate concentrations, and total phosphate concentrations were routinely monitored in each of the chambers. Replicate chambers were treated with Prudhoe crude oil alone (natural degradation), Prudhoe crude oil plus cyanide (abiotic degradation) or Prudhoe crude oil plus oleophilic nitrogen (CRNF, Sun Oil Co.) and phosphorus (octylphosphate) fertilizer (stimulated degradation). Numbers of oil degraders and total heterotrophs were determined periodically in each of the growth chambers by plate count procedures previously reported (Horowitz and Atlas, 1977a). Sufficient replicates of each treatment were established to permit periodic sacrificing of duplicate chambers for oil extraction and analysis. Oil was recovered at 2 week intervals for 2 months during the initial summer and then after approximately 1 year following winter. No samples were recovered during the winter. Residual oil was recovered by solvent extraction with diethyl ether. Recovered oil was dried with sodium sulfate, weighed and analysed by gas liquid and column chromatography. The chromatographic analyses

were as previously described, except that 80-100 mesh chromosorb W was used as the solid support for the gas chromatographic columns.

After 30 days exposure in the flow-through system, the adaptation of the microbial communities in the variously treated growth chambers to the presence of oil and the relative abilities of the microbial communities to degrade branched and straight chain alkanes was estimated. Twenty ml samples were collected from the growth chambers and placed in biometer flasks. NH_4Cl (10 mM), $NaNO_3$ (10 mM), Na_2HPO_4 (0.5 mM) and 50 µl of Prudhoe crude oil spiked with either ^{14}C n-hexadecane (sp. act. 4.0 µCi/ml) or ^{14}C-pristane (sp. act. 0.17 µCi/ml) were added. After incubation for 1 week at 10 C, the oil was recovered by ether extraction and fractionated by column chromatography (Horowitz and Atlas, 1977b). The ^{14}C in the paraffinic fraction was measured by liquid scintillation counting.

NARL Gasoline Spill

Microorganisms were enumerated from sediment in the freshwater lake that had been contaminated 1 year earlier by the accidental leaded gasoline spillage at NARL (Horowitz and Atlas, 1977b). Total viable heterotrophs were enumerated on trypticase soy agar supplemented with 0.10% unleaded gasoline. Gasoline hydrocarbon utilizing, lead tolerant, microorganisms were enumerated on Bushnell Haas Agar with 0.5% MOGAS (leaded gasoline). Ratios of gasoline utilizers to viable heterotrophs were calculated. Enumerations were performed weekly for 9 weeks at 5 sites in the lake.

Under Ice

For modelling under ice oil spillages, stainless steel cylinders, 10cm by 20cm were implanted into the bottomside of the ice. One ml Prudhoe Bay crude oil was injected into the cylinders by scuba divers.

The cylinders were held in place *In situ* with styrofoam flotation collars. Under ice experiments were conducted during May. Oil was recovered periodically from replicate cylinders with diethyl ether in seperatory funnels. Ice was allowed to melt before extraction. Recovered oil was analysed by gas liquid chromatography as described above.

In Sediment

For modelling oil in sediment, 25cm by 25cm by 7cm trays were filled with freshly collected sediment. Benthic animals were removed from most of the trays. Two hundred ml Prudhoe Bay crude oil was added to the sediment and the trays replaced *In situ*. The trays were placed at a depth of approximately 5m. During transport of the trays to and from the bottom, by scuba divers, a Plixiglas lid was held over the trays. Oil in sediment experiments were begun in May. Periodically replicate trays were recovered for analysis. For recovery of oil from sediment, sediment was mixed with a glass stirring rod to evenly distribute the residual oil. Five sediment samples, ca 150g each, were removed from each tray. Solvent (diethyl ether) was repeatedly mixed with sediment samples and decanted. Solvent extracts were concentrated under an air stream,dried with sodium sulfate and brought to constant volume. Recovered oils are being analysed by gas liquid chromatography as described above.

Recolonization of oil contaminated sediment by benthic invertebrates was studied by recovering animals from the exposed,oil treated and control sediment trays. A sieve, 4mm hole diameter was used for recovering animals. Recovered animals were identified and quantitated. Multiple replicate trays were used for each determination.

Sublethal Effects of Oil on Amphipods in Sediment

Amphipods (Boeckosimus affinus) collected in Elson Lagoon were placed in 15cm by 40cm x 10cm chambers. Two cm sediment (2500g) was placed in each chamber. Some of the sediment was saturated with fresh Prudhoe Bay crude oil. Some of the sediment was saturated with weathered (ca 25% weight reduction under an air stream) Prudhoe Bay crude oil. Five exposure regimes were used: No oil in sediment (control); 50% sediment area no oil, 50% sediment area fresh Prudhoe crude oil; 100% sediment area fresh Prudhoe Bay crude oil; 50% sediment area no oil, 50% sediment area weathered Prudhoe Bay crude oil; 100% sediment area contaminated with weathered Prudhoe Bay crude oil. Twenty-five amphipods were tested in each chamber. Each chamber was run in duplicate. Seawater was replaced every 2 weeks.

Mortality was checked daily. Every 2 weeks the response of surviving amphipods to food, movement activity and burrowing activity was measured. Burrowing was measured by determining the number of amphipods located under the sediment surface. The location of burrowed amphipods was recorded as in oil contaminated or uncontaminated sediment. Also the locations of amphipods that were not burrowed were recorded.

Movement was measured in an open field apparatus, a square tray, 50cm on a side and 10cm deep, with a grid marked on the bottom. The grid was composed of 100 squares, each 5cm on a side. Each square was identified by a letter and a number, e.g. A3, L6. After ten minutes acclimation, each animal was given a gentle touch with a glass rod. As the animal moved, the grid square it occupied was noted every five

seconds for three minutes. For quantitative purposes an animal was presumed to be in the center of the square at the time of recording. An animal was assumed to travel in a straight line between squares. The distance traveled for each five second interval was then determined and expressed as units of movement (units of movement x 5 = cm moved).

The ability to find and recognize food was measured in a glass tray, similar to the open field tray. Animals were placed in the tray and after ten minutes acclimation, a cube of fresh meat, approximately 2cm on a side, was placed in the middle of the tray. The number of animals feeding was noted at ten minute intervals for one hour.

Numerical Taxonomy of Hydrocarbon Utilizing Bacteria

Bacteria enumerated on Pudhoe Bay crude oil agar from the flow through control and oil exposed chambers (Horowitz and Atlas, 1977a) gasoline agar from sediment and water in the accidentally contaminated freshwater lake at NARL (Horowitz and Atlas, 1977b) were randomly selected for numerical taxonomic testing. A total of 243 bacteria were selected. Table 1 shows the sources of the organisms selected. Approximately 127 phenotypic characteristics were determined for each strain. Characterization included morphological, physiological, biochemical, nutritional and antibiotic sensitivity testing. Incubation was at 4C unless otherwise indicated. Table 2 shows the list of features determined.

For morphological observations young bacterial cultures (1-4 day depending on growth rate), grown on marine agar 2216 slants overlain with 1 ml Rila marine salts solution, were used. Wet mounts were used for observing motility, cell shape, size and arrangement. Gram staining and acid fast staining (Ziehl Neelsen method) were performed (Society of American Bacteriologists, 1957). Ten day cultures grown

on marine agar 2216 were used for observation of colonial morphology, including colony size and shape. Production of diffusible and non-diffusible pigments were also observed. Fluorescent pigments were checked with a UV lamp (260 nm).

Physiological characteristics tested included salt, temperature. and pH tolerance ranges. All incubations for physiological tests were up to two weeks in duration.

For temperature testing replicate inoculated marine agar 2216 plates were incubated at 5, 10, 15, 20, 25, 37 and 43°C. For pH testing replicate marine agar 2216 plates were adjusted with HCl or NaOH to pH 3, 4, 5, 6, 7, 8, 9 and 10. For salt tolerance testing NaCl was added to a base medium (bacto-tryptone, 0.5%; Bacto-yeast extract 0.1%; $FeCl_3.6H_2O$, 0.01%; $NH_4 NO_3$, 0.00016%; $NaHPO_4$, 0.0008%; Bacto Agar, 15%; pH8.0) to give 0, 3, 5, 7.5, 10 and 15% NaCl.

Production of catalase was detected with 3% H_2O_2 on 10 day marine agar 2216 grown cultures. Cytochrome oxidase was detected on 10 day cultures according to the method of Gaby and Hadley (Skerman, 1967) with time for development of blue color limited to 1 minute. Methyl red tests were run on 14 day cultures grown in MR-VP broth (Difco) prepared with full strength Rila marine salts solution (Society of American Bacteriologists, 1957).

For reduction of nitrate and nitrite, nitrate broth (Difco) with full strength Rila marine mix was used. The naphthylamine-sulfanilic acid test was used to detect nitrite and zinc dust to detect residual nitrate (Skerman, 1967).

Production of acid from maltose, fructose, galactose, lactose, sucrose, glycerol and mannitol was detected using MOF medium (Difco) with 1% substrate incubated for 14 days. Oxidative/fermatative tests were performed using MOF medium supplemented with 1% glucose (Hugh and Leifson, 1953) incubated for 14 days.

Hydrolysis of starch, was tested after 7 days incubation on marine agar 2216 plates with 0.5% potato starch. The plates were flooded with Lugol's iodine and clear zones around colonies were scored as positive tests. For lipase activity, hydrolysis of tween 20 and tween 80 was used (Sierra, 1957). One % tween in marine agar 2216 with 0.01% $CaCl_2$ incubated for 14 days was used. For hydrolysis of gelatin, 10% gelatin was added to marine agar 2216. After 7-10 days incubation the plates were flooded with acid $HgCl_2$ and clear halos recorded as positive tests (Skerman, 1967).

Antibiotic sensitivity was tested by spreading suspensions on marine agar 2216 plates and applying BBL (Cockeyville MD) antibiotic discs (albamycin 5 µg; gantrisin, 5 µg; nitrofurantoin 100 µg; pencillin G, 2 units; strepomycin 2 µg). After 14 days incubation, zones of inibition were measured and sensitivity determined against standard inhibition zones (BBL).

Lead tolerance was determined using marine agar 2216 with 0.2% lead acetate. Mercury tolerance was determined using marine agar 2216 with 0.2% mecuric chloride. Growth (colony formation) within 14 days incubation was recorded as tolerance to the respective heavy metal

Nutritional characterization was accomplished by testing utilization of 52 substrates as source of carbon and energy. Vitamins were added

as growth factors. The media for substrate utilization tests was prepared as follows: Portion 1; KH_2PO_4, 0.1 g; Trizma base, 6.0 g; NH_4NO_3, 1.0 g; $FeCl_3.6H_2O$, 0.005 g; 500 ml Rila marine salts solution; pH adjusted with HCl to 8.0. Portion 2; Purified agar (Difco), 10 g; 500 ml distilled H_2O. Portions 1 and 2 were autoclaved separately and mixed at 45°C. Vitamins were added as growth factors (thiamine 1 µg; pantothenate 1 µg; riboflavin 1 µg; nicotinic acid 1 µg; choline 1 µg; pyridoxamine 1 µg; cyanocobalamine 1 µg; folic acid 0.05 µg; p amino-benzoate 0.05 µg; biotin 0.05 µg per 1).

Substrates were sterilized by autoclaving or filter sterilization according to the methods of Stanier et al. (1966). Hydrocarbons were sterilized by sonication. Substrates were mixed with basal media just before pouring to yield final concentrations of 0.1% for all substrates except carbohydrates 0.15% and phenol 0.0125%. All substrate utilization tests were innoculated with a multiple syringe inoculator (Kaneko et al, 1977). Positive growth was determined after 14 days incubation by visual reference to control plates of the same basal medium with no added substrate.

Data were coded in binary form using a 1 for positive, 0 for negative and blank for undetermined characters. Data were entered according to RKC format (Rogosa et al. 1971). Data were punched on cards, verified and proofread by 2 individuals. Data were initially processed with the CREATE computer program (Krichevsky, 1977) which subjects the data to extensive checking for format conformation, date duplication and other errors. A report was generated using the QUERY computer program (Krichevsky, 1977) so that the data were in the proper format for the numerical taxonomy program TAXAN 5. Using the TAXAN 5 program,

Jaccard similarity coefficients (S_J) were generated. Cluster analyses were performed using unweighted average linkage sorting of Jaccard coefficients (Sokal and Sneath, 1963). Clusters of strains with similarities greater than 68% were designated as taxonomic groupings. The input data were backsorted according to the ordering of strains in the cluster triangle and the feature frequencies of all characteristics determined using the feature analysis program FREAK.

RESULTS

Continuous Flow System

The temperature and salinity fluctuations during the initial summer are shown in Fig. 1. After 10 days of the experiment, there was an abrupt rise in temperature and salinity. After approximately 70 days, the temperature had lowered to the point where the chambers froze and flow stopped. No further temperature of salinity measurements were made after this point.

The average temperatures and salinity values were higher during this summer (1976) than during the previous summer (1975). Also, the total phosphate levels (30-100 µg atom P/l) and available nitrogen levels (20-80 µg atom N/l as NH_4^+ and NO_3^-) in the water were especially high during this summer. Dissolved oxygen levels were saturated throughout the summer in all growth chambers. The pH of the water at the beginning of the experiment was approximately 6-7 with a gradual rise to 8-8.5. Addition of oleophilic fertilizers at the beginning of the experiment caused an immediate drop in pH to 3.6 with a gradual rise back to pH 6 taking a few days. Repetition of fertilizer addition after the pH of the water had reached 8.5 led to a drop only to pH 7 with a rapid rise back to pH 8.5.

The number of colony forming units of both oil degraders and total heterotrophs increased in response to oil addition (Fig. 2). Greater increases were found when oleophilic fertilizers were added together with oil. In most cases, fluctuations in population levels were parallel in control (no oil), oil treated, and oil plus fertilizer treated chambers; fluctuations were of greater magnitude in the latter chambers. No significant differences were found in numbers of psychrophiles-psychrotrophs enumerated at 5 C (Fig. 2) and mesophiles enumerated at 20 C (not shown in Figs.).

Tests after 30 days exposure to crude oil also showed that microbial populations had adapted to the presence of the oil and that the oil had enriched for effective hydrocarbon utilizing microorganisms (Table 3). Compared to controls, approximately 30% of both ^{14}C hexadecane and ^{14}C pristane were lost when incubated with water from control chambers that had not been previously exposed to oil. Prior exposure to oil resulted in increased losses to approximately 50% for both pristane and hexadecane. Similar losses were found whether or not fertilizer had been added together with the oil. The indication is that pristane and hexadecane are degraded at similar rates.

The greatest weight losses of oil from the growth chambers occured during the first 2 weeks (Table 4). The poisoned controls showed that 18-19% of the added oil was lost abiotically during the initial summer. Without added fertilizer, an additional 6% of the oil was lost by biodegradation. With added fertilizer, the biodegradative loss was increased to about 22%. No further oil losses occured during the winter while the system was frozen.

The gas chromatographic analysis showed, as previously found (Atlas, 1975; Horowitz and Atlas, 1977a), that residual oil contained similar relative percentages of resolved hydrocarbons, regardless of whether biodegradative losses were prevented by addition of KCN or stimulated by addition of fertilizer (Table 5). All exposed oils showed loss of low molecular weight hydrocarbons found in fresh oil. Following these initial losses, neither time of exposure, treatment, nor amount of degradative weight loss altered the percent composition of the resolved hydrocarbons in the residual oil.

The column chromatographic analyses showed that abiotic losses lowered the relative percentages of paraffins, monoaromatic and diaromatic fractions and increased the percentages of polyaromatic and polar compounds (Fig. 3). As with the gas chromatographic analyses, further biodegradative losses did not result in changes in hydrocarbon class composition regardless of time of exposure, treatment or amount of degradative loss.

NARL Gasoline Spill

The ratios of gasoline utilizers to total heterotrophs were high at all sites (Table 6). Ratios of less than 0.1 are typical background levels. Ratios of greater than 0.1 generally indicate hydrocarbon contamination. The area of contamination had spread from the previous summer. Only sampling time 5 did not show evidence of contamination. It appears that gasoline contamination persists in this lake and is widespread 1 year after the spillage.

Under Ice

When spilled under ice, there were only slow compositional changes (Fig. 4). Only 75% of the weight of the oil was recoverable 1 day after

spillage of oil under ice. We believe that the loss was physical, probably occuring during handling, transport and recovery of the ice cores. The residual oil from the under-ice experiments, showed a gradual disappearance of light hydrocarbons. However, even after 3 weeks exposure, significant quantities of light hydrocarbons remained in the oil. The ice cover probably limits evaporative losses and dissolution probably accounts for the loss of light hydrocarbons. If the toxic fraction of crude oil resides in this light fraction, oil spilled under ice will retain toxic properties for prolonged periods. Poor ice conditions prevented sampling of oil under ice for longer exposure periods.

In Sediment

In sediment, there was a 45% weight loss after 60 days compared with oil remaining after 2 days. Technical problems prevented recovery of oil from contaminated sediment at 0 time. Even after 2 days, light hydrocarbons were not found in the oil recovered from the sediment (Fig. 5). During 60 days of exposure, there were major changes in the composition of oil in sediment. These changes are probably due to biodegradation. It is likely, however, that petroleum hydrocarbons will remain in contaminated sediment for a long time. When exposed for a similar 2 month period, greater concentrations of hydrocarbons of low molecular weight remained in oil exposed in sediment than in oil exposed on water. These sediment studies are on-going, to examine the long term fate of oil in Arctic marine sediments.

We found that application of Prudhoe crude oil to sediment resulted in rapid death or movement from oil contaminated sediment of the indigenous benthic invertebrates. To simulate this observed situation, we studied the recolonization of oil contaminated and unoiled sedi-

ment, following physical removal of the invertebrate organisms. Within 1 week, attempts at recolonization by invertebrates was observed. Several organisms, e.g., a burrowing anemone (unidentified sp.) and a bivalve (Liocyma sp.), were found in both control and oil contaminated trays. These organisms died, however, within 2 weeks in the oil contaminated sediment, but remained alive for over 1 month in the unoiled sediment. After 60 days, neither the bivalve nor the anemone was found in control or oil contaminated trays. We are unable to explain the disappearance of these organisms from the control trays at the 60 day sampling time. The 60 day samples were collected shortly after several severe storms, which may have disturbed the benthic community.

The 60 day samples did show some interesting differences in the recolonization of oiled and control sediment (Table 3). Amphipod species were found much less frequently in oil contaminated than control sediments. Contact with Prudhoe Bay crude oil has previously been found to be toxic to several different amphipods from this area (Busdosh and Atlas, 1977). Isopods were found as frequently in oil contaminated as control sediment. Percy and Mullin (1975), reported that the isopod found in our study shows little preference for oil tainted or untainted sediments and is extremely resistant to crude oil dispersions. With respect to polychaetes, some polychaete species were found in oil contaminated, but not control sediments and one species found in controls was absent from oil contaminated sediment. Some polychaete species may be attracted to oil while oil is toxic to other species. Carr and Reish (1977) found the degree of susceptibility of polychaetes to petroleum to be species dependent. Overall, oil contaminated sediment is recolonized by invertebrates within 2 months

of contamination. The benthic community composition, however, is markedly different in oil contaminated than control sediment 60 days after contamination. Thus, while relatively rapid "recovery" is likely following oil contamination of Arctic benthic ecosystems, relatively long lasting changes in the benthos can be predicted.

Sublethal Effects of Oil on Amphipods in Sediment

When sediment was contaminated with fresh Prudhoe Bay crude Oil, burrowing activity of the amphipod Boeckosimus (= Onisimus) affinis was reduced for a one month period, during which the oil underwent weathering changes (Table 8). Exposure to weathered oil did not inhibit burrowing activity. Given a choice (50% oil contaminated/50% uncontaminated), the amphipods selectively burrowed into the uncontaminated sediment. This preference extended beyond 1 month. Exposure in experimental chambers to sediment contaminated with fresh oil also resulted in decreased movement and feeding (Table 9) activities during the month that the oil underwent initial weathering. Exposure to weathered oil did not adversely affect feeding or movement activities. Mortality rates were low for amphipods exposed to sediment contaminated with fresh or weathered oil (Table 10). Behavioral changes in feeding and movement appear to be temporary and associated with the light hydrocarbons present in fresh crude oil.

Numerical Taxonomic Studies

The Jaccard coefficients were calculated both using hydrocarbons as features and omitting hydrocarbons as features. The simplified dendrograms for the cluster analyses are shown in figures 6 and 7. There were some differences between the analyses. There were fewer clusters and the clusters tended to be larger when hydrocarbons were not included

in the analysis, i.e. hydrocarbon utilization tests tended to split groups. Overall,however, there was good correlation between the two analyses. The major clusters appeared in both analyses and were approximately the same size. Approximately half of the isolates fell into a single cluster. The remainder fell into smaller clusters many of which contained only 2-3 organisms. There were a few medium size clusters of 10-20 organisms. Interestingly the isolates in the largest cluster came from multiple sources (Tables 11 and 12). All of the gasoline spill isolates were in this cluster. Also this cluster contained isolates from the flow through system . These results indicate that very closely related organisms flourished in response to a crude oil spill in marine water's with and without fertilizers added and to a leaded gasoline spill in a freshwater lake in both sediment and water. The identity of this cluster of organisms remains to be determined.

DISCUSSION

Addition of oil to Arctic coastal waters resulted in rapid increases in numbers of microorganisms. The highest numbers of microorganisms were found in the first 2-3 weeks following addition of oil especially when fertilizer was added together with oil. Ratios of oil degraders to total heterotrophs were 5 times higher under oil slicks than in controls. The greatest rate of oil degradation also occured during this period.

Application of oleophilic fertilizer resulted in lowered pH especially during the period of high ice melt when buffering capacity was apparently particularly low. The drop in pH could have initially been bacteriocidal, but within 1 week, numbers of microorganisms were the same or higher in fertilizer treated growth chambers as in chambers where only oil was added. In fact, the greatest biodegradative losses occurred during the first 3 weeks when fertilizer was added. Fertilizer additon has previously been found not to increase abiotic losses (Horowitz and Atlas, 1977a). Some concern is certainly warranted about adding octyl phosphate to poorly buffered ecosystems.

Compared to the previous summer, degradative losses were higher during summer 1976 than 1975, but degradative losses were still relatively low. Summer 1975 had been especially harsh with severe ice conditions. During summer 1976, nutrient levels in the water and average temperature were higher than the previous year. The relatively low rates of degradative loss during summer cannot be attributed entirely to limiting temperatures, concentrations of nitrogen and phosphorus, or oil composition. It is possible that other nutrients became limiting following the first few weeks of oil exposure. It is also possible

that bacterial populations that colonize oil droplets (Horowitz, et al., 1975) prevent diffusion of nutrients and oxygen to the oil, effectively preventing further rapid degradation. Lack of oil spreading could also have contributed to limited surface area available for degradation. This possibility could also explain why recovered oil showed the same relative composition regardless of the extent of degradation.

Allowing the oil to freeze into the ice matrix effectively models what would naturally occur to oil floating on the surface during the period of ice formation. In fact, in nearshore regions where oil may concentrate along the beach, ice freezes to the bottom Degradation looses under these conditions during winter were found to be nil. Higher rates of degradation could occur for oil trapped under ice. Obviously, annual rates of oil dissappearance are much lower than would be predicted from only summer studies. Oil contaminating Arctic coastal waters may persist for many years.

Degradation of oil under ice appears to be restricted. The ice cover seems to prevent evaporative losses. This implies that a toxic fraction of light hydrocarbons will persist for a prolonged period under ice. The rates of biodegradation under ice were very low.

Analysis of oil residues in sediment have not yet been completed. The initial analyses show some biodegradative losses. The recolonization sutdies indicate that some organisms are attracted to the oiled area, some are indifferent, and some avoid the oil contaminated areas. Laboratory studies with amphipods showed that they avoided burrowing in oil contaminated sediment. These laboratory experiments also showed that

oil retained a fraction for about 1 month that caused behavioral changes in movement and feeding activities. This fraction was not lethal and was lost after 1 month as shown by a return to behavior typical of control animals.

The numerical taxonomy of bacteria isolated from hydrocarbon contaminated areas showed that the same group of organisms was enriched for in freshwater by leaded gasoline and in marine ecosystems by crude oil. This group of organisms was tolerant of heavy metals and capable of degrading a wide range of hydrocarbons. The implication of this result is that a single seed culture of bacteria can be found that could be applied to radically different petroleum hydrocarbon mixtures in radically different ecosystems. This possibility requires further study. The applicability of seeding oil slicks to enhance biodegradation may be possible in restricted ecosystems such as those in these Arctic studies.

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PERSONNEL

In addition to the Principal Investigator, Dr. Ronald M. Atlas, major contributions to this project were made by Mr. Amikam Horowitz and Mr. Michael Busdosh. Both Mr. Horowits and Mr. Busdosh are doctoral candidates at the University of Louisville.

PLANS FOR FUTURE

We plan to complete the analyses of the oil recovered from the In situ sediment exposure. We also plan to complete biodegradation potential analyses of samples impacted by the 1976 NARL gasoline spillage. We also plan to try to identify compounds found to accumulate following the gasoline spillage. Data on amphipod response to hydrocarbons will be further analysed. Also further interpretation of the taxonomic analyses will be accomplished. Any further field work will be dependent on ONR providing logistic support. Further field work could include additional recovery of oil from contaminated sediment both from the site of the NARL gasoline spill and the experimental Prudhoe crude oil spill site in Elson Lagoon.

Table 1. Sources of organisms used in cluster analyses.

Flow-Through System	
Control (No Oil)	33
Oil	75
Oil plus Fertilizer	75
NARL Gasoline Spill	
Water	30
Sediment	30

TABLE 2 Features for numerical taxonomic testing.

*RKC#	FEATURE
012021:	Gram positive.
012022:	Gram negative.
012009:	Cells are acid fast by Ziehl-Neelsen method.
013001:	Cells motile.
003008:	Cells are rod-shaped.
003001:	Cells are spherical.
003023:	Pleomorphic cells are characteristic.
015001:	Cells occur singly.
015002:	Cells occur in pairs.
015003:	Cells arranged in angular fashion after division (snapping).
015004:	Cells occur in chains.
015005:	Cells arranged in irregular aggregates.
015006:	Cells arranged in two-dimensional tetrads.
015017:	Organisms filamentous.
020001:	Colonies are pure (paper) white on solid medium.
020002:	Colonies are gray on solid medium.
020019:	Diffusible (water-soluble) pigments are produced.
020038:	Non-diffusible red pigments are produced.
020039:	Non-diffusible brown pigments are produced.
020041:	Non-diffusible violet (purple) pigments are produced.
020043:	Non-diffusible golden (yellow) pigments are produced.
020044:	Non-diffusible orange pigments are produced.
020057:	Non-diffusible black pigments are produced.
020060:	Fluorescent pigment observable with short wavelength ultraviolet light (ca. 260 nm.).
098001:	Non-diffusible pink pigments are produced.
016005:	Agar macro-colonies are translucent.
016006:	Agar macro-colonies are transparent.
016007:	Agar macro-colonies are opaque.
016008:	Agar macro-colony margin is entire.
016027:	Colony surface is glistening.
016028:	Colony surface is dull (matte).
019168:	Sensitive to nitrofurantoin concentration (disc) 100 ugm.
019210:	Senistive to penicillin G concentration (disc) 2 units.
019233:	Sensitive to streptomycin concentration (disc) 2.0 ugm.
019486:	Sensitive to novobiocin (albamycin) concentration (disc) 5 ugm.
019490:	Sensitive to sulfisoxazole (gantrisin) concentration (disc) 5 ugm.
016057:	Growth takes place at an initial pH of 4.0.
016056:	Growth takes place at an initial pH of 5.0.
016055:	Growth takes place at an initial pH of 6.0.
016054:	Growth takes place at an initial pH of 7.0.
016187:	Growth takes place at an initial pH of 8.0.
016053:	Growth takes place at an initial pH of 9.0.
016194:	Growth takes place at an initial pH of 10.0.
017032:	Growth at 5 C.
017012:	Growth at 10 C.
017013:	Growth at 15 C.

*Rogosa, et al. (1971)

TABLE 2 - continued

*RKC#	FEATURE
017037:	Growth at 20 C.
017014:	Growth at 25 C.
017015:	Growth at 37 C.
017045:	Growth at 43 C.
018028:	Added NaCl is required for growth.
018003:	Growth in the presence of 0.5% NaCl.
018004:	Growth in the presence of 3% NaCl.
018022:	Growth in the presence of 7.5% NaCl.
018008:	Growth in the presence of 10% NaCl.
024009:	Gelatin is hydrolyzed (liquefied).
025357:	Starch is hydrolyzed.
032020:	Tween 20 is hydrolyzed.
032023:	Tween 80 is hydrolyzed.
024164:	Hydrogen peroxide is decomposed.
024248:	Kovacs' oxidase test positive.
024185:	Methyl red test is positive.
024138:	Nitrate is reduced.
024210:	Nitrite is reduced.
024014:	D-Glucose catabolized aerobically.
024015:	D-Glucose catabolized anaerobically.
025193:	Acid produced from D-Fructose.
025212:	Acid produced from Lactose.
025213:	Acid produced from Maltose.
025215:	Acid produced from Sucrose.
026351:	Acid is produced from 1, 2, 3-Propanetriol (Glycerol).
026371:	Acid is produced from D-Mannitol.
025194:	Acid produced from D-Galactose.
025020:	D-Galactose is utilized.
025021:	D-Glucose is utilized.
025038:	Lactose is utilized.
025041:	Sucrose is utilized.
026005:	Ethanol is utilized.
026045:	1, 2, 3-Propanetriol (Glycerol) is utilized.
026065:	D-Mannitol is utilized.
026075:	Cyclohexanol is utilized.
026076:	Meso-Inositol is utilized.
026089:	Phenol is utilized.
028002:	Acetic acid is utilized.
028011:	Palmitic acid is utilized.
028027:	Succinic acid is utilized.
028036:	10-Octadecanoic acid is utilized.
028037:	Oleic acid is utilized.
028066:	Citric acid is utilized.
028078:	Benzoic acid is utilized.
028668:	Cyclohexane carboxylic acid is utilized.
029015:	L-Asparagine is utilized.
029016:	L-Aspartic Acid is utilized.
029023:	L-Glutamic Acid is utilized.
029036:	L-Methionine is utilized.

TABLE 2 - continued

*RKC#	FEATURE
029047:	L-Tryptophan is utilized.
029051:	L-Valine is utilized.
030144:	Ethanolamine is utilized.
031093:	Cyclohexanone is utilized.
034143:	Urease (3.5.1.5) is produced.
031101:	n-Decane is utilized.
031108:	n-Hexadecane is utilized.
031112:	n-Nonane is utilized.
031113:	n-Octadecane is utilized.
031115:	n-Pentadecane is utilized.
031166:	1-Methylnaphthalene is utilized.
031172:	Omega-Phenyldecane is utilized.
031178:	Toluene is utilized.
031590:	Pristane (2,6,10,14-Tetra-methylpentadecane) is utilized.
031602:	Pentadecylcyclohexane is utilized.
031638:	2,2,4,4,6,8,8-Heptamethylnonane is utilized.
031674:	Ethylcyclohexane is utilized.
031686:	Dicyclohexyl is utilized.
031710:	Diphenylmethane is utilized.
031746:	Acenaphthalene is utilized.
031752:	9-Methylanthracene is utilized.
098100:	Naphanol is utilized.
098101:	Prudhoe crude oil is utilized.
098102:	JP5 is utilized.
098103:	Gasoline (unleaded) is utilized.
098104:	Mineral oil is utilized.
098105:	API Reference Oil #1 is utilized.
098106:	API Reference Oil #2 is utilized.
098107:	API Reference Oil #3 is utilized.
098108:	API Reference Oil #4 is utilized.
098197:	Tolerant to lead.
098198:	Tolerant to mercury.

Table 3 Percent of ^{14}C Hydrocarbons Remaining Relative to Sterile Controls After 1 Week Incubation at 10 C.

	Hexadecane	Pristane
Control (no oil previously added)	70.3%	71.2%
Exposed to oil for 30 days	56.7%	47.5%
Exposed to oil + fertilizers for 30 days	54.9%	47.5%

Table 4 Percent Loss of Crude Oil From Model Flow-Through System.

Time in Days	Crude Oil	Crude Oil + Fertilizers
15	17	30
30	22	36
45	24	36
60	25	38
70	26	40
340	25	40

Table 5 Gas Chromatographic Analysis of Residual Oils Showing Average Relative Percentages of the Resolved Compounds in Residual Oils Recovered Periodically During Approximately 1 Year Exposure.

Retention Time (min)	Fresh Crude Oil	S.D.	C.O. + KCN	S.D.	C.O.	S.D.	C.O. + Fert.	S.D.	Identified Paraffins
<16.21	61.3	<.1	-	-	-	-	-	-	
16.22	5.2	<.1	-	-	0.5	1.6	-	-	
17.37	1.4	<.1	-	-	-	-	-	-	
17.67	1.8	<.1	-	-	-	-	-	-	
18.55	4.8	<.1	5.0	2.1	4.7	2.0	4.7	2.0	$n\text{-}C_{12}$
20.01	3.7	<.1	3.1	0.9	2.4	1.6	1.2	1.8	
20.62	4.3	<.1	9.7	0.3	10.1	1.6	9.8	1.4	$n\text{-}C_{14}$
21.57	0.6	<.1	2.3	0.0	2.6	0.5	2.7	0.8	
21.97	0.3	<.1	-	-	-	-	-	-	
22.53	4.6	<.1	16.4	0.5	17.3	1.3	17.1	1.6	$n\text{-}C_{15}$
23.48	0.6	<.1	-	-	0.2	0.6	0.8	1.5	
24.27	2.3	<.1	10.5	0.5	11.0	1.5	10.6	1.3	$n\text{-}C_{16}$
24.85	0.7	<.1	4.0	0.6	3.9	1.0	4.4	1.1	
25.91	3.8	<.1	18.3	0.8	20.1	2.2	20.2	2.0	$n\text{-}C_{17}$ + pristane
27.51	3.0	<.1	14.3	0.9	15.7	1.8	15.7	1.6	$n\text{-}C_{18}$
29.01	0.1	<.1	-	-	-	-	-	-	
29.60	1.7	<.1	10.4	0.8	9.4	1.5	9.8	1.2	$n\text{-}C_{19}$
32.31	0.4	<.1	6.2	0.8	1.8	1.9	2.4	1.8	$n\text{-}C_{20}$

S.D. = standard deviation
C.O. = Prudhoe crude oil

TABLE 6 Ratio of gasoline utilizers to total heterotrophs.

SAMPLE TIME	SITE 1	2	3	5	11
1	0.733	1.200	1.116	0.389	-
2	0.061	0.048	0.162	0.224	0.229
3	0.109	0.300	0.262	0.119	0.200
4	0.846	0.746	0.917	0.805	0.468
5	0.002	0.005	0.034	0.004	0.020
6	1.284	0.924	-	-	-
7	0.632	1.420	1.878	0.354	0.361
8	0.431	0.292	0.478	0.698	0.472
9	0.294	0.335	0.382	0.563	0.673

TABLE 7 Benthic Recolonization 60 days after Oil Contamination

	SPECIES	Mean #/square meter CONTROL	OIL
	Pontoporeia femorata	16	6
Amphipoda	*Aceroides latipes*	16	0
	Melita sp. (dentata?)	14	1
	Gammaracanthus loricatus	2	0
	Monoculodes sp.	20	4
Isopoda	*Saduria entomon*	4	5
	Acanthostephieia behrengiensis	4	0
Polychaeta	*Pectinaria granulosa*	0	5
	Nephythys longosetosa	0	4
	Spio sp.	0	4

TABLE 8 Burrowing Activity (% burrowed).

WEEKS EXPOSURE	CONTROL (no oil)	100% Fresh Oil	50% Fresh Oil		100% Weathered Oil	50% Weathered Oil	
			Uncontaminated area	Contaminated area		Uncontaminated area	Contaminated area
2	80	24	43	17	26	85	9
4	85	19	33	29	27	64	18
6	87	51	43	38	8	56	18
8	73	43	48	43	11	40	22
10	85	38	51	24	2	51	28
12	92	53	56	27	0	53	42
16	92	61	61	32	4	45	31

TABLE 9 Feeding Activity (% feeding)

WEEKS EXPOSURE	CONTROL (no oil)	100% Fresh Oil	50% Fresh Oil	100% Weathered Oil	100% Weathered Oil
2	95	24	36	68	94
4	98	65	55	60	89
6	100	85	100	92	98
8	100	93	100	91	96
10	98	85	95	89	95
12	92	100	95	93	100
16	100	97	98	93	95

TABLE 10 Mortality (# surviving).

WEEKS EXPOSURE	CONTROL (no oil)	100% Fresh Oil	100% Fresh Oil	100% Weathered Oil	100% Weathered Oil
0	50	50	50	50	50
2	48	50	47	50	47
4	47	43	42	48	45
6	47	41	42	48	45
8	47	40	42	47	45
10	47	40	41	45	43
12	45	38	41	45	43
16	45	38	41	45	42

TABLE 11 Sources of clusters run without hydrocarbons.

Cluster	Total #	Other	NARL Gasoline Spill Sediment	NARL Gasoline Spill Water	Flow Through Sea Water	Flow Through Oil	Flow Through Oil & Fertilizer
1	107	2	30	30	5	16	24
1a	3						3
1b	19		16	2			1
1c	12		11				1
1d	27		2	25			
1e	15	1			2	8	4
1f	3						3
1g	9						3
1h	3					2	1
1i	3				3		
2	2				2		
3	2				1	1	
4	2				1		1
5	2					2	
6	2					2	
7	15					1	14
8	26	1			2	15	8
8a	5				1	4	
8b	11					8	3
8c	4				1		3
9	7					6	1
10	15				1	7	7
10a	4					1	3
11	3				3		
12	14					10	4
12a	5					4	1
13	4				4		
14	3					3	
15	2				2		
16	3					2	1
17	7				5		2
17a	5				5		
18	2					1	1
19	3					2	1
20	2					1	1
21	2						2

TABLE 12 Sources of clusters run with hydrocarbons.

Cluster	Total #	Other	NARL Gasoline Spill Sediment	NARL Gasoline Spill Water	Flow Through Sea Water	Flow Through Oil	Flow Through Oil & Fertilizer
1	110	2	30	30	6	19	23
1a	14	1			2	8	3
1b	13					2	11
1c	6						6
1d	60		30	29			1
2	3				1		2
3	9					5	4
4	3					3	
5	2					2	
6	2					2	
7	6					5	1
8	2						2
9	5						5
10	4				4		
11	3				3		
12	2				2		
13	3					1	2
14	2						2
15	2						2
16	8						8
17	3					3	
18	22				2	13	7
19	2				2		
20	8					7	1
21	2					1	1
22	5				5		
23	3					2	1
24	2					1	1
28a	4				1	3	
28b	10					7	3

FIGURE LEGENDS

Fig. 1. Temperature and salinity fluctuations during the initial summer.

Fig. 2. Viable counts of psychrophilic-psychrotrophic bacteria.

A. Oil degrading bacteria

B. Total heterotrophic bacteria

□ = control, with no added oil or fertilizers

▲ = with added oil

Δ = with added oil and oleophilic fertilizers

Fig. 3. Column chromatographic analysis.

1. Fresh crude oil

2. Average of exposed oils

a. Paraffinic fraction

b. Monoaromatic fraction

c. Diaromatic fraction

d. Polyaromatic polar fraction #1

e. Polyaromatic polar fraction #2

The middle bars represent the standard deviations.

Fig. 4. Gas liquid chromatographic analysis of oil spilled under ice.

A. Prudhoe Bay crude oil

B. residual oil after 1 day exposure

C. residual oil after 5 days exposure

D. residual oil after 14 days exposure

E. residual oil after 21 days exposure

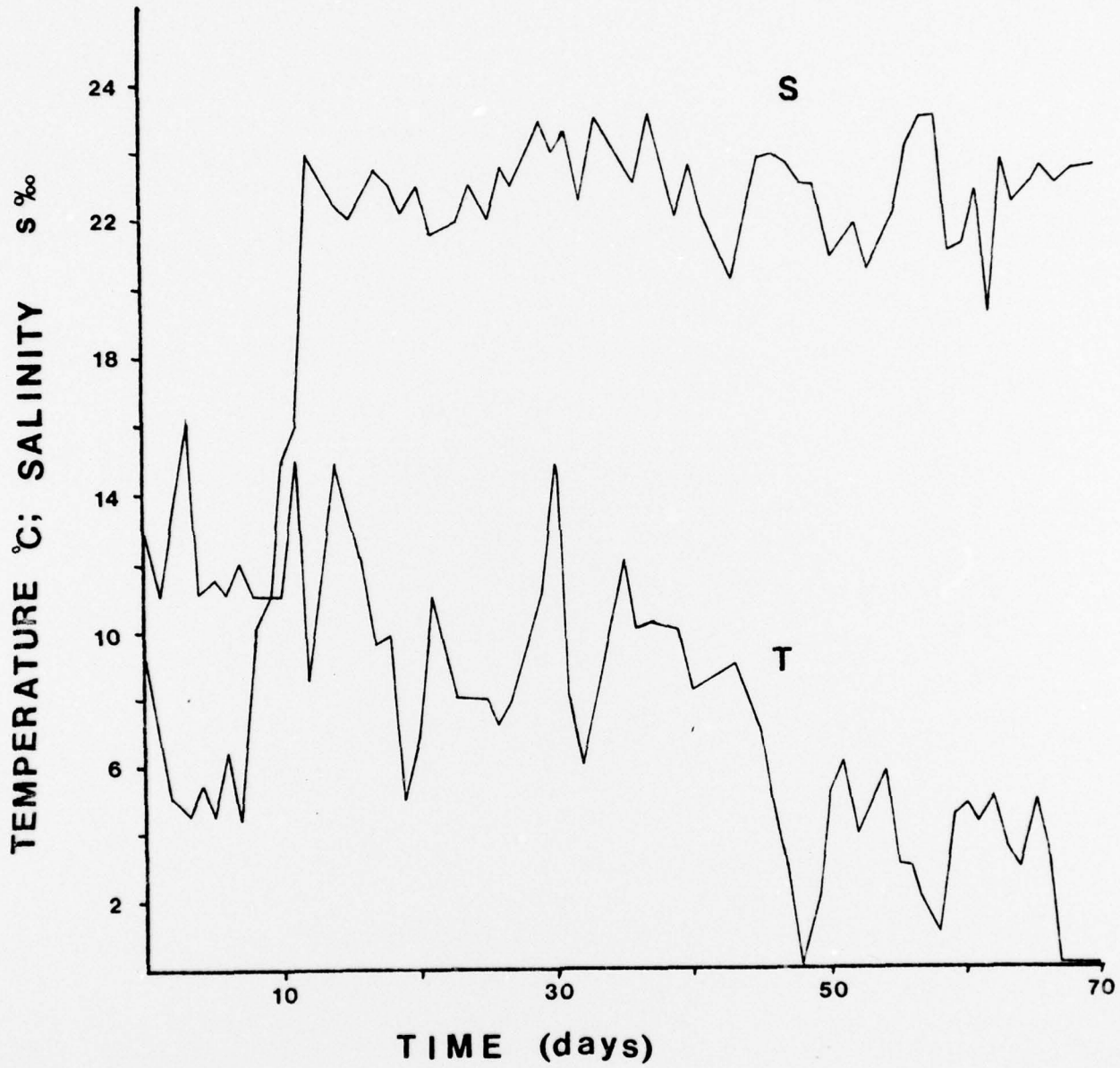
Fig. 5. Gas liquid chromatographic analysis of oil spilled in sediment.

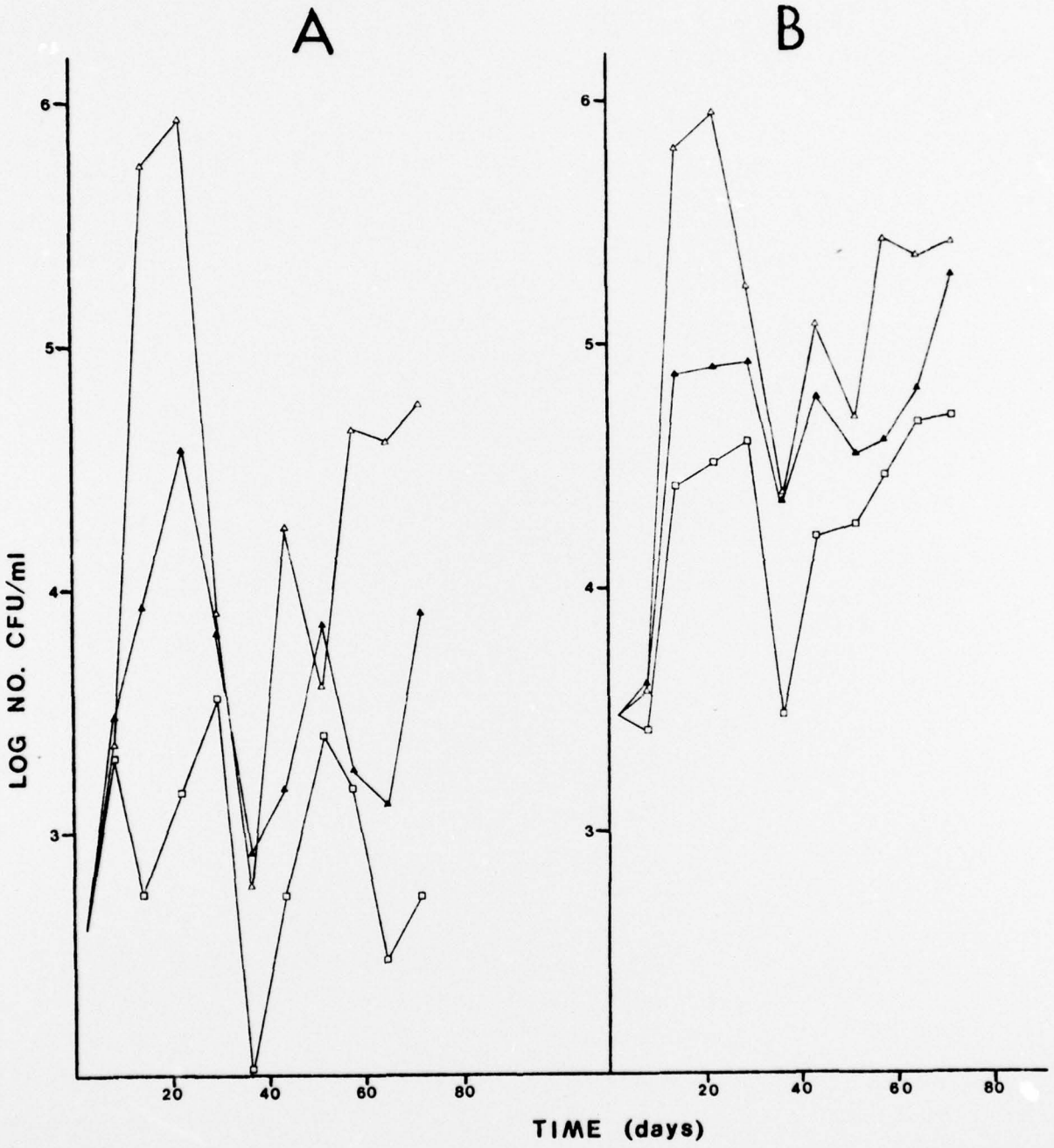
A. residual oil after 2 days

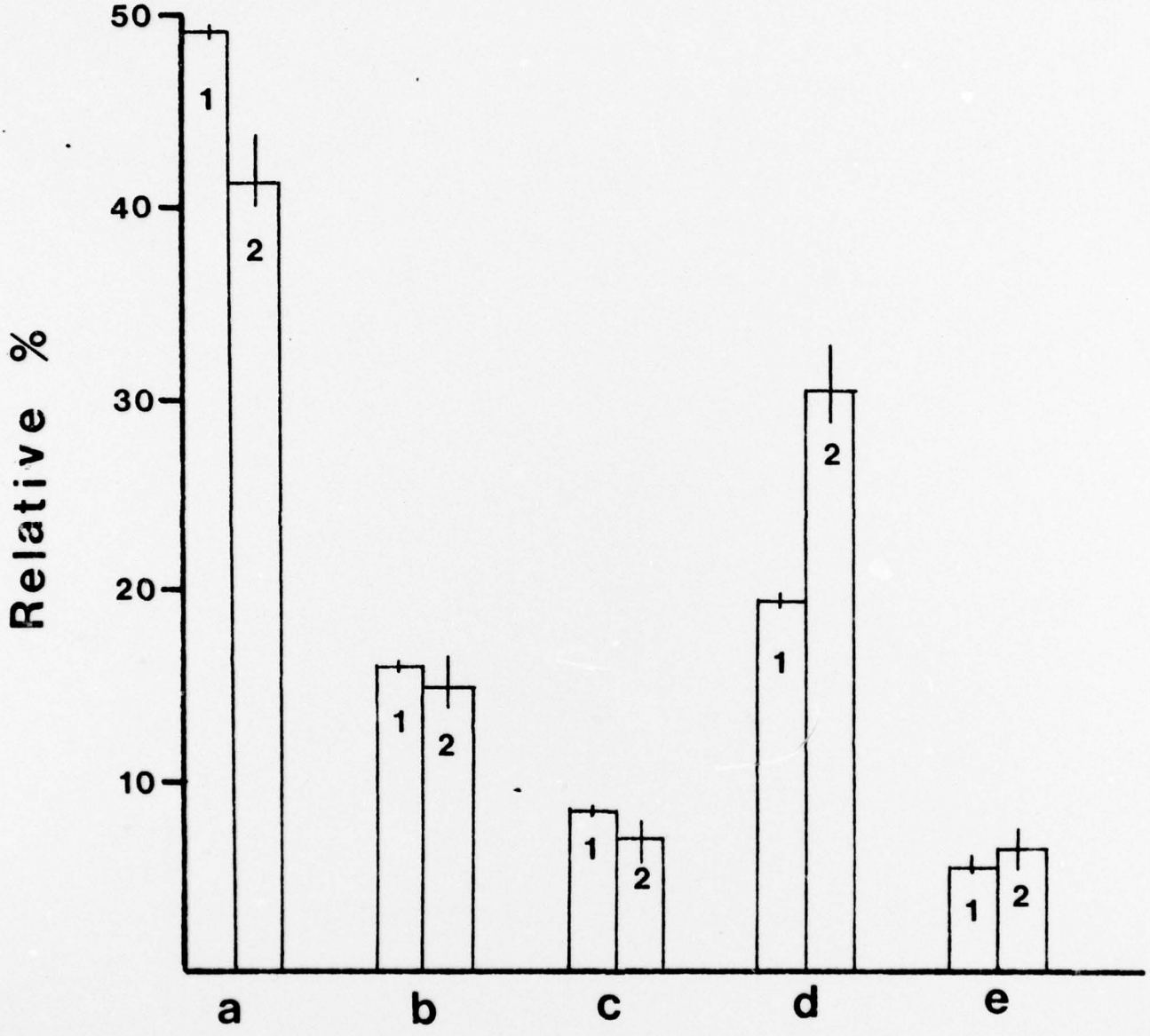
B. residual oil after 60 days

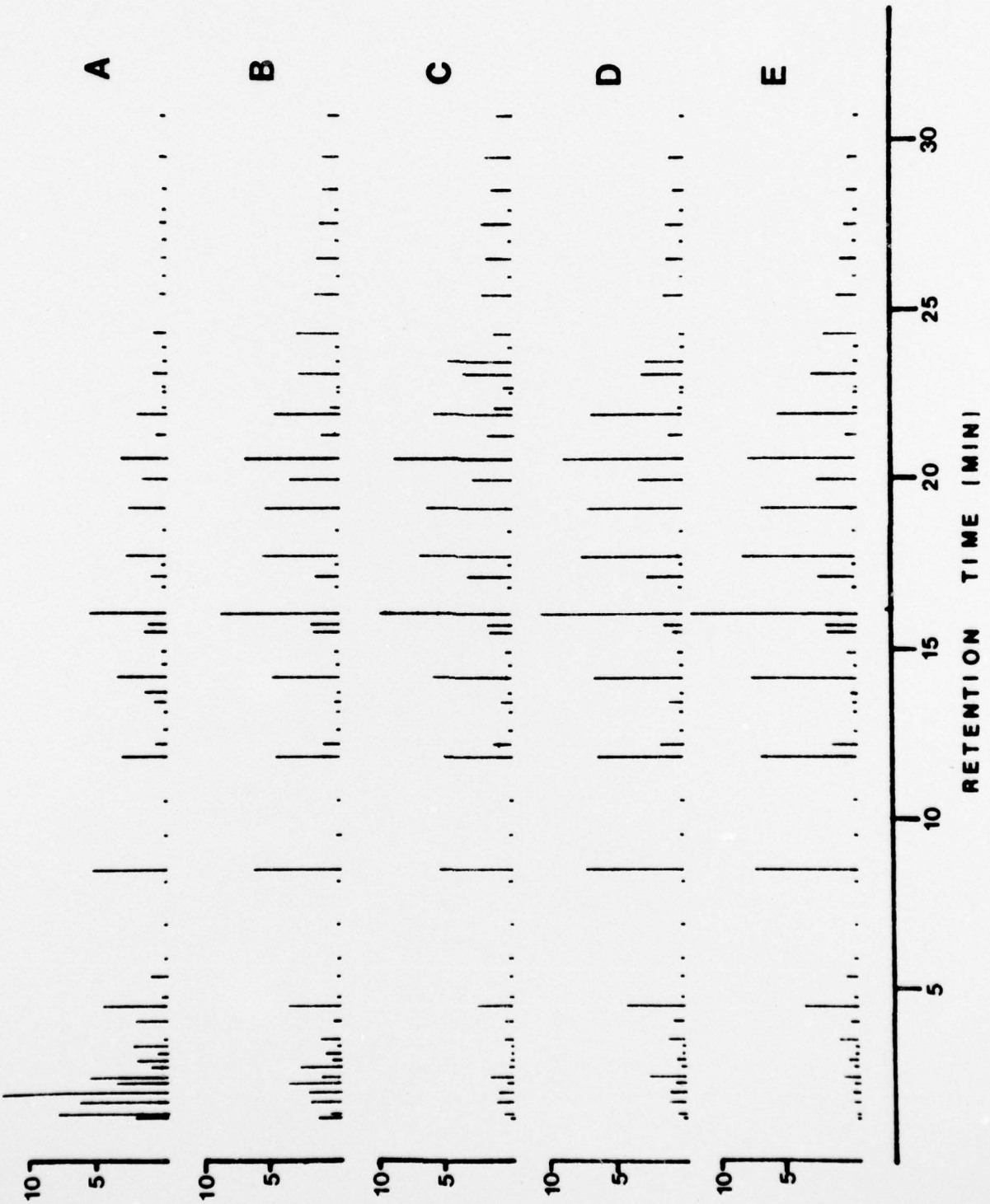
Fig. 6 Simplified dendrogram of cluster analysis run without hydrocarbons as features - unweighted average of Jaccard coefficients.

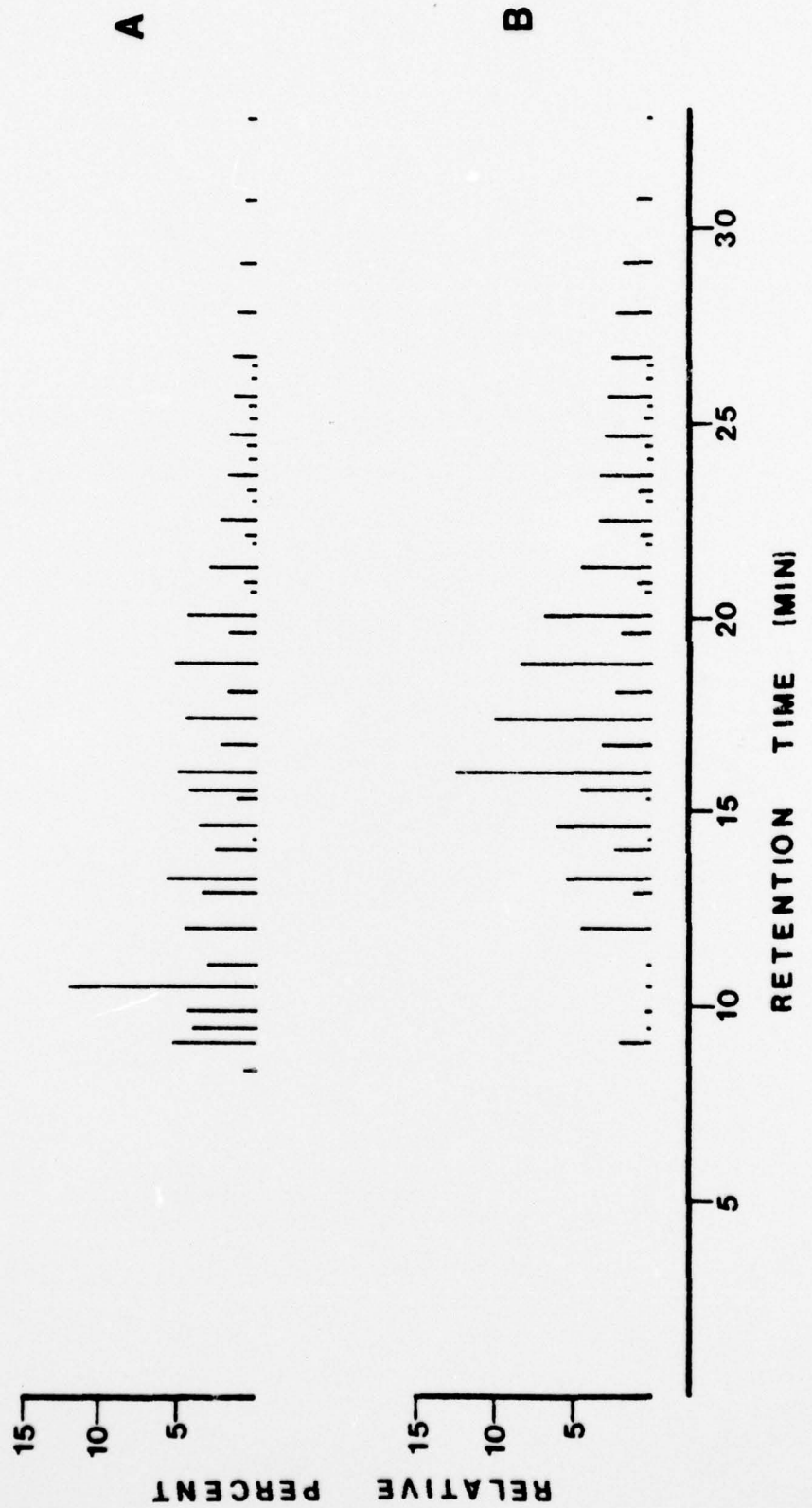
Fig. 7. Simplified dendrogram of cluster analysis run with hydrocarbons as features - unweighted average of Jaccard coefficients.

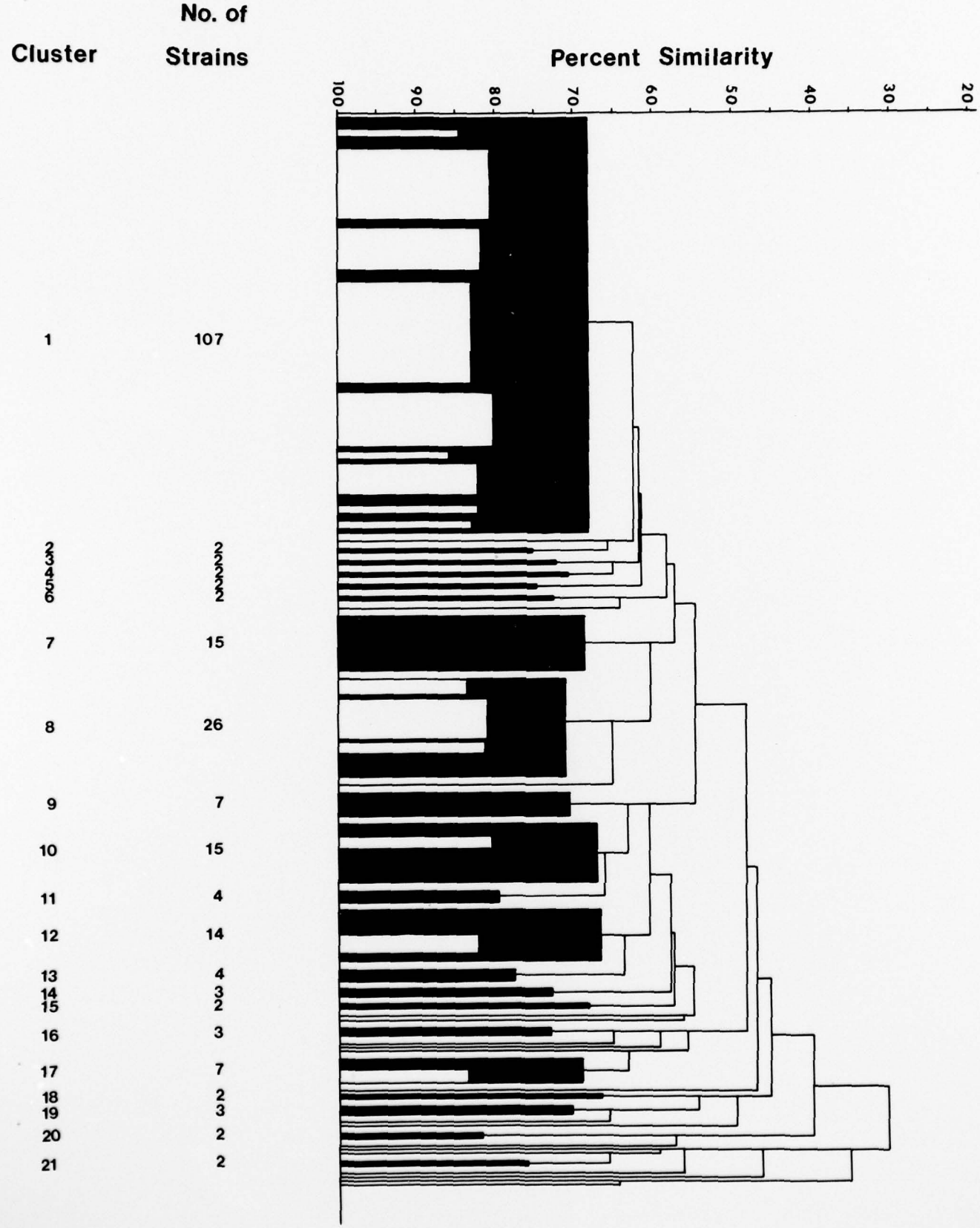

TEMPERATURE °C; SALINITY s ‰
24
22
18
14
10
6
2
S
T
10
30
50
70
TIME (days)


A
B
6
5
4
3
LOG NO. CFU/ml
20
40
60
80
TIME (days)


Relative %
50
40
30
20
10
1
2
a
b
c
d
e


A
B
C
D
E
10
5
RELATIVE PERCENT
5
10
15
20
25
30
RETENTION TIME (MIN)


A
B
15
10
5
RELATIVE PERCENT
5
10
15
20
25
30
RETENTION TIME (MIN)


Cluster
No. of Strains
Percent Similarity
100
90
80
70
60
50
40
30
20
1 107
2 2
3 2
4 2
5 2
6 2
7 15
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10 15
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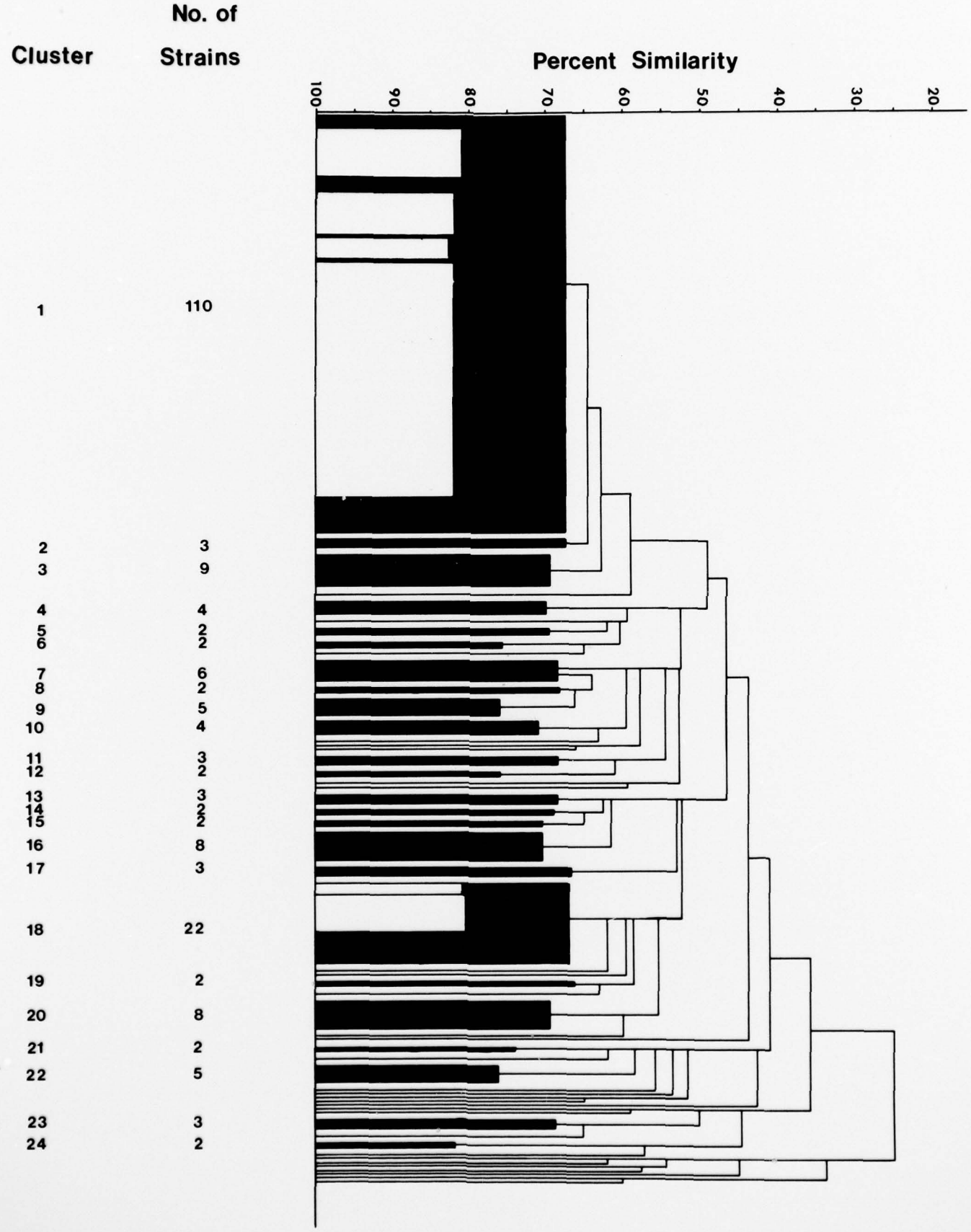
14 3
15 2
16 3
17 7
18 2
19 3
20 2
21 2


Cluster
No. of Strains
Percent Similarity
100
90
80
70
60
50
40
30
20
1
110
2
3
3
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4
4
5
2
6
2
7
6
8
2
9
5
10
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11
3
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14
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15
2
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18
22
19
2
20
8
21
2
22
5
23
3
24
2

PUBLICATIONS RESULTING FROM THE PROJECT TO DATE

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